How does HIV-1 infect a host cell?

HIV infects the T helper cell because it has the CD4 receptor and the chemokine receptor on its surface. In order for HIV-1 to infect the cell it requires fusion of viral and cellular membranes. Membrane fusion is mediated by the viral envelope structure of gp160. Gp160 forms a homotrimer and undergoes glycosylation within the Golgi apparatus. It is then cleaved by a cellular protease into
gp120 and gp41. Gp41 contains a transmembrane domain and remains in a trimeric configuration. Gp120 contains most of the external domains of the envelope glycoprotein complex. (Kwong *et al*)

Gp120 interacts with the CD4 receptor and a chemokine receptor on the cell. Once gp120 interacts the upper segments of gp120 change configuration to allow fusion proteins to open and be inserted into target membrane to form prehairpin intermediate. Click here to see a PDB of 3-D structure of HIV-1 gp120, complexed to CD4 and a neutralizing antibody. You must have CHIME plug-in or can download it.

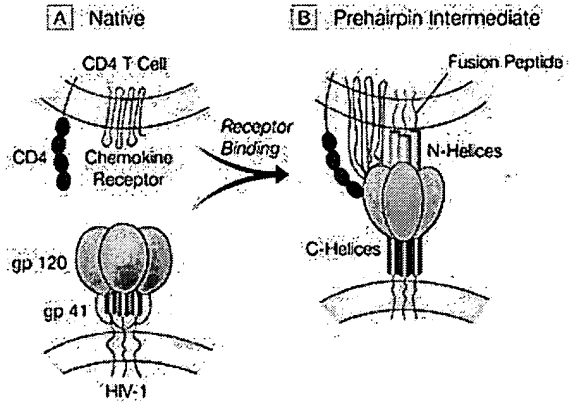
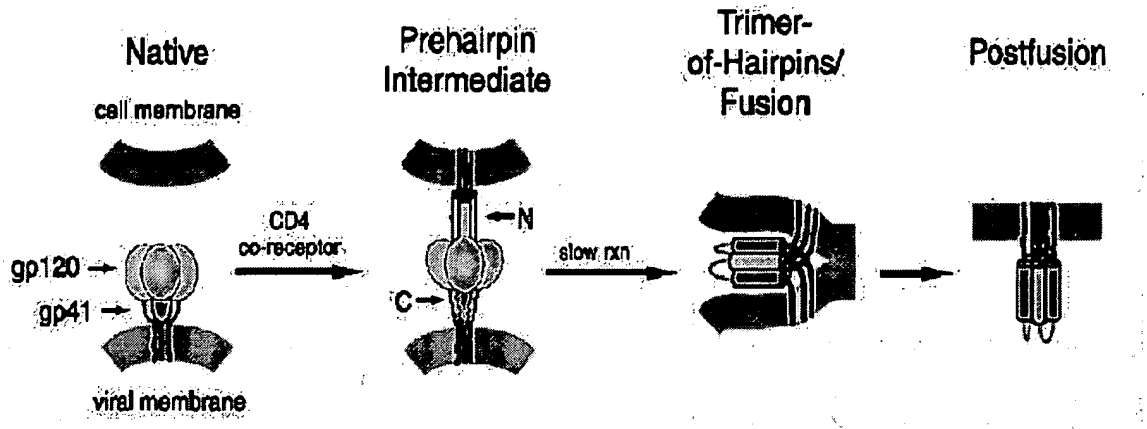


Figure taken from D'Souza MP *et al*
A) gp120 interaction with chemokine receptor and CD4 receptor. B) Conformational change occurs and the fusion peptides of the gp41 become inserted into the cell membrane (prehairpin intermediate). C- and N- helices exposed but not yet associated with one another.

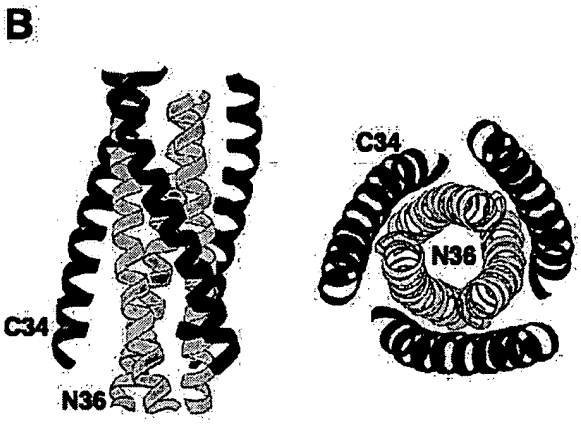
Prehairpin intermediate resolves to the fusion active hairpin structure when the C and N peptides interact creating the trimer

of hairpins structure in gp41.

Figures below taken from Root *et al*



Trimer-of-hairpins structure of gp41 Root *et al*



Trimer of hairpins structure consists of an outside coil and an inside coil. Outside coil is 3 alpha helices formed by C-terminal regions of gp41 molecules packed anti-parallel against inside coil. The inside coil is a 3 stranded coiled coil formed by N-terminal regions of gp41 molecules.

Home Background of HIV/AIDS Stages of HIV Infection
How Does HIV infect host cells?
How can HIV-1 Entry be blocked? Protein Design of Entry Inhibitors
Future for Entry Inhibitors in HIV Drugs

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Expression, Purification, and Characterization of gp160e, the Soluble, Trimeric Ectodomain of the Simian Immunodeficiency Virus Envelope Glycoprotein, gp160*

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The envelope glycoprotein, gp160, of simian immunodeficiency virus (SIV) shares 25% sequence identity with gp160 from the human immunodeficiency virus, type I, indicating a close structural similarity. As a result of binding to cell surface CD4 and co-receptor (CCR5 and CXCR4), both SIV and human immunodeficiency virus gp160 mediate viral entry by membrane fusion. We report here the characterization of gp160e, the soluble ectodomain of SIV gp160. The ectodomain has been expressed in both insect cells and Chinese hamster ovary (CHO)-Lec3.2.8.1 cells, deficient in enzymes necessary for synthesizing complex oligosaccharides. Both the primary and a secondary proteolytic cleavage sites between the gp120 and gp41 subunits of gp160 were mutated to prevent cleavage and shedding of gp120. The purified, soluble glycoprotein is shown to be trimeric by chemical cross-linking, gel filtration chromatography, and analytical ultracentrifugation. It forms soluble, tight complexes with soluble CD4 and a number of Fab fragments from neutralizing monoclonal antibodies. Soluble complexes were also produced of enzymatically deglycosylated gp160e and of gp160e variants with deletions in the variable segments.

The SIV and HIV envelope glycoproteins, known as gp160, bind to cell surface receptors, effect cell entry by fusion of viral and cellular membranes, and as major surface antigens, induce neutralizing antibodies in the host. gp160 is synthesized as a single chain precursor, which is cleaved after oligomerization by furin, or a similar enzyme, into the two chains gp120 and gp41. Cleavage occurs during transport to the plasma membrane (1, 2). In the cleaved gp120/gp41 molecule, gp120 and gp41 are associated noncovalently. The gp120 polypeptide contains the binding sites for the receptor CD4 (3-5) and the chemokine binding co-receptor (6-10). gp41 contains an N-terminal nonpolar fusion peptide, a nonpolar transmembrane anchor, and a C-terminal cytoplasmic domain (11-13).

The interaction of the gp120 polypeptide of gp120/gp41 with CD4 causes a conformational change in the viral glycoprotein, inducing altered antibody reactivity, increased proteolytic susceptibility, and the tendency in some viral strains for gp120 to dissociate from gp41 (14-16). The conformational change caused by CD4 binding apparently increases the affinity of gp120/gp41 for the chemokine-binding co-receptor (17-18). Interaction with the chemokine receptor activates the membrane fusion activity of gp120/gp41, probably by triggering a conformational change in gp41. This change is likely to expose the N-terminal fusion peptide and to refold gp41 into a helical hairpin, thereby placing the fusion peptides and transmembrane anchors of gp41 at the same end of a rod-shaped molecule (19-24).

There is a formal similarity among the SIV and HIV-1 gp160 glycoproteins, the envelope glycoproteins of other retroviruses such as Moloney murine leukemia virus, and HTLV-1, the Gp glycoprotein of filoviruses, and the hemagglutinin (HA) of influenza virus. Each is synthesized as a single chain precursor, which after cleavage results in an N-terminal subunit (gp120, SU, Gp1, HA1) with receptor binding activity and a C-terminal, membrane-anchored subunit (gp41, TM, Gp2, HA2) having a nonpolar, glycine-rich N-terminal fusion peptide followed by heptad repeats characteristic of -helical coiled-coils (reviewed in Refs. 25 and 26). Evidence suggests that these viral glycoproteins are all trimeric (27-32), although some early studies proposed dimers or tetramers for gp160 (33-35). In each case, the N-terminal subunit (gp120, SU, Gp1, HA1) is monomeric when prepared in the absence of the transmembrane subunit, but oligomeric in its presence (36, 37). The C-terminal subunits are all trimeric when expressed in the absence of the N-terminal polypeptides, and they adopt similar structures dominated by an N-terminal, central triple-stranded -helical coiled-coil

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[1] The abbreviations used are: SIV, simian immunodeficiency virus; HIV, human immunodeficiency virus; HA, hemagglutinin; mAb, monoclonal antibody; PCR, polymerase chain reaction; bp, base pair; kb, kilobase pair; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; EGS, ethylene glycol bis(succinimidylsuccinate); CHO, Chinese hamster ovary; Endo H, endoglycosidase H.

and an outer layer of three helices or extended strands packing antiparallel along the central coiled-coil and connected to it by a loop with a reverse (reviewed in Ref. 26).

The three-dimensional structure of an enzymatically deglycosylated fragment of monomeric gp120 from HIV-1, with the variable segments V1, V2, and V3 deleted, along with 52 residues at the N terminus and 19 at the C terminus, has been determined by x-ray crystallography. The crystals contain a

complex of truncated gp120 with a two-domain fragment of CD4 and an Fab fragment from the monoclonal antibody 17b (38). This structure contains about 60% of the gp120 residues (39, 40). It is in the CD4-induced conformation, which has enhanced affinity for the monoclonal antibody 17b (41). The structure of the gp120 trimer has not been determined, although it has been modeled (42), and the conformation of gp120 as it would appear on virus before CD4 binding is currently unknown.

We report the development of expression systems using insect and mammalian cells for production of soluble, oligomeric ectodomain, gp160e, from the SIV envelope glycoprotein. Purified oligomers are shown to bind CD4 and Fab fragments of neutralizing monoclonal antibodies. Experiments show that the molecule is trimeric. These observations should facilitate biochemical and structural characterization of the viral glycoprotein in its native, oligomeric form.

EXPERIMENTAL PROCEDURES

SIV gp160e Expression in CHO Cells- SIV gp160e from the SIV strain Mac32H pJ5 (43) was expressed in CHO-Lec3.2.8.1 cells using the expression construct pSIV-M (44) following a procedure described previously (45). Briefly, 20 g of *Sal* I-linearized pSIV-M DNA was transfected into the Lec3.2.8.1 cells by calcium phosphate precipitation (Stratagene). Transformants were selected in GMEM medium (Life Technologies, Inc.) containing 25 M methionine sulfoximine and assayed for secretion of soluble SIV gp160e by enzyme-linked immunosorbent assays. Immulon plates (Dynatech Laboratories Inc.) were coated with anti-SIV ENV monoclonal antibody KK19 (46) by incubating a 4 g/ml solution overnight at 4 °C; the plates were then blocked with 1% bovine serum albumin in PBS at room temperature for 2 h. Cell culture supernatant (50 l) from each transformed clone was added to the plates and incubated overnight at 4 °C. The plates were washed with 0.1% bovine serum albumin in PBS and incubated with 0.2 g/ml biotinylated mAb KK41 (47) for 2 h at room temperature. After washing again, the plates were developed with horseradish peroxidase-conjugated streptavidin (Sigma). The highest expressing cells were chosen for rescreening with the same assay. The expression of SIV gp160e was also confirmed by immunoprecipitation. Large scale production of SIV gp160e was carried out following the protocol previously described (45) in 175-cm [2] tissue culture flasks or 850-cm [2] roller bottles (Corning).

The protein expressed from pSIV-M in Lec3.2.8.1 cells was found to have two N termini (see Fig. 1). To produce SIV gp160e variants with the V1, V2, and V3 (gp160e (V1V2V3)) or just the V1 and V2 (gp160e (V1V2)) segments deleted and a corrected N terminus in the Lec3.2.8.1 cells, a shorter version of the tissue plasminogen activator leader sequence was fused with the N terminus of SIV gp160. pSIV-M was amplified by PCR with primers BC-16 (5 -TAGTCTCATTGACCATGTCT-3) and BC-45 (5 -GCTGTGTGCTGCTGCTGTGTGGAGCAGTCTTCGTTTCGCCCAGAGCTAGCACTCAATATGTCACAGTC-3). The resulting 424-bp PCR product was gel-purified and reamplified with BC-16 and BC-44 (5 -GCTCTAGAAGGGACGCTGTGAAGCAATCATGGATGCAATGAAGAGAGGGCTCTGCTGTGTGCTGCTGCTGTGT-3) to give a 477-bp fragment, which was subsequently digested with *Xba* I and *Nsi* I and gel-purified. This fragment was ligated with a 1.4-kb *Nsi* I- *Eco* RI fragment from pFBSIV V1V2 (see below) or a 1.4-kb *Nsi* I- *Eco* RI fragment from pFBSIV V1V2V3 (see below) and then cloned into pEE14 (48) digested with *Xba* I and *Eco* RI to generate pNES9 V1V2 and pNES1 V1V2V3, respectively. Expression of pNES9 V1V2 and pNES1 V1V2V3 followed the same procedure except that transformants were were screened with different mAbs, as mAb KK9 (46) and biotinylated mAb KK41 used for pNES9 V1V2, mAb 2C3 [2] and biotinylated mAb KK41 used for pNES1 V1V2V3.

gp160e coding sequence. The PCR product was digested with *Bam* HI and *Eco* RI, gel-purified, and cloned into pMelBac (Invitrogen) to generate an intermediate construct pBacSIV-5, which was sequenced using primers BC-1, BC-2, BC-6 (5 -CACCAACGGCAGCATCAA-3), BC-7 (5 -AGATGTAATGACACAAAT-3), BC-8 (5 -AAATTGGAAGGATGCAAT-3), BC-9 (5 -GAGACCTCACGTGTAACT-3), BC-10 (5 -GTGCAGCAACAGCAACAG-3), and BC-16 to verify the entire sequence of SIV gp160e open reading frame. pBacSIV-5 was then amplified using primers BC-14 (5 -TTTATGGTCGTATACATTTCTTACATCTATGCGACTCAATATGTCACAGTC-3) and BC-16. The resulting PCR product was reamplified by BC-13 (5 -CGCGGATCCATGAAATTCTTAGTCAACGTTGCCCTTGTTTTTATGGTCGTATACATT-3) and BC-16 to produce a 447-bp fragment, which was digested with *Bam* HI and *Nsi* I, gel-purified, and designated as fragment 1. This fragment allows fusion of the N terminus of SIV gp160e and the honeybee melittin secretion signal sequence. pBacSIV-5 was digested with *Nsi* I and *Nco* I and gel-purified to give a 1.5-kb fragment, designated as fragment 2, which encompassed a major portion of SIV gp160e open reading frame. To introduce a histidine tag at the C terminus of SIV gp160e, a 757-bp PCR product was amplified using pBacSIV-5 as template and BC-9 and BC-24 (5 -CCGGAATTCTCAATGATGATGATGATGAGTGCGACCTTCGATTTGTATATACTTTATCCAAGA-3) as primers and digested with *Nco* I and *Eco* RI, gel-purified, and designated as fragment 3. Fragments 1, 2, and 3 were ligated together and cloned into pFastBac-1 (Life Technologies, Inc.), digested with *Bam* HI and *Eco* RI, to give the expression construct pFBSIV-His1. Constructs were also made to express SIV gp160e variants with deletions of segments V1, V2, and V3; of V1 and V2 only; or of V3 only. pFBSIV-His1 was amplified by PCR with primers BC-2 and BC-32 (5 -CCATTATGCATTGGAGCAGGTCACTGTAACACTTCTATTAT-3), which replaces V1 and V2 segments with a short linker GAG. The PCR product was digested with *Nsi* I and *Nco* I, gel-purified, and inserted into pFBSIV-His1, which was digested with *Nsi* I and *Nco* I to generate pFBSIV V1V2-His1. pFBSIV V3-His3 was constructed by overlapping PCR. pBacSIV-5 was amplified with primers BC-1 and BC-43 (5 -ACACCAACCTGCTCCTCTACATTTCATTGTTAGATT-3) and primers BC-18 (5 -TTGTATATACTTTATCCAAGAAGCAAG-3) and BC-42 (5 -TGTAGAGGAGCAGGTTGGTGTTGGTTTGGAGGAAAT-3) to produce two overlapped 892-bp and 1.0-kb fragments, respectively. These two fragments were gel-purified, mixed, and reamplified with primers BC-1 and BC-18 to give a 1.9-kb fragment in which the V3 segment was replaced with GAG linker. This fragment was then digested with *Nsi* I and *Nco* I and cloned into pFBSIV-His1, which was also digested with *Nsi* I and *Nco* I to generate pFBSIV V3-His3.

To construct pFBSIV V1V2V3-His1, pFBSIV V3-His3 was used as a template to produce a 1.4-kb PCR fragment with primers BC-2 and BC-32 to replace V1 and V2 segments with a GAG. The PCR product was digested with *Nsi* I and *Nco* I, gel-purified, and inserted into pFBSIV-His1, which was digested with *Nsi* I and *Nco* I to generate pFBSIV V1V2V3-His1. pFBSIV V1V2 and pFBSIV V1V2V3 were also made to express gp160e variants without histidine tags. pBacSIV-5 was used as a template for PCR with primers BC-2 and BC-10. The PCR product was then digested with *Nco* I and *Eco* RI, gel-purified, and inserted into *Nco* I- and *Eco* RI-digested pFBSIV V3-His3 and pFBSIV V1V2V3-His1, respectively, to yield pFBSIV V1V2 and pFBSIV V1V2V3.

Both restriction digestion and DNA sequencing verified all of the expression constructs.

Recombinant baculovirus was generated according to the manufacturer's protocol and amplified in Sf9 insect cells in TNM-FH medium (Sigma). Expression of SIV gp160e was confirmed by a Western blot using sheep antisera against SIV envelope glycoprotein. The optimal amount of virus and postinfection harvest time was determined by small scale tests in 100-ml spinner flasks. Infected *Trichophusia ni* (Hi-5) cells were found to secrete significantly more SIV gp160e than Sf9 cells, judging by Western blot. For large scale protein production, 10

lated mAb KK41 for pNES9 V1V2 by enzyme-linked immunosorbent assays.

SIV gp160e Expression in Insect Cells- pFBSIV-His1 was constructed to express SIV gp160e in insect cells using the Bac-to-Bac expression system (Life Technologies, Inc.). The pSIV-M was amplified by PCR using primers BC-1 (5 -CGCGGATCCGACTCAATATGTCACAGTCTTTTAT-3) and BC-2 (5 -GGCCGAATTCTATATCCAAGAAGCAAGG-3) to produce a DNA fragment encompassing the complete SIV

liters of *T. ni* (Hi-5) cells (2 10 ^{6} cells/ml) in Ex-Cell 405 medium (JRH Biosciences) were infected at a multiplicity of infection of 2.5. 3 days after infection, the supernatant was harvested by centrifugation and concentrated to 1 liter in a tangential flow filtration system, ProFlux M 12 (Millipore Corp.).

Purification of SIV gp160e- The CHO Lec3.2.8.1 supernatants containing secreted SIV gp160e were harvested by centrifugation or by filtration through a Corning filter (0.22 mm). SIV gp160e was purified by an immunoaffinity chromatography using a mAb 17A11 [3] affinity column (5-ml bed volume), where the monoclonal antibody 17A11 was

[2] M. Kim, B. Chen, R. E. Hussey, Y. Chishti, D. Montefiori, J. A. Hoxie, D. C. Wiley, S. C. Harrison and E. L. Reinherz, manuscript in preparation.

[3] J. A. Hoxie,unpublished results.

cross-linked at 5 mg/ml to GammaBind Plus Sepharose (Amersham Pharmacia Biotech) with dimethyl pimelimidate (Pierce). The supernatants were passed through the column with a flow rate of about 0.5 ml/min. After extensive washing with PBS, the protein was then eluted with 100 m M glycine (pH 3.0), followed by immediate neutralization with 2 M Tris-HCl (pH 8.0). The fractions were analyzed by SDS-PAGE. The fractions containing SIV gp160e were pooled, concentrated, and further purified by gel filtration chromatography on Superdex 200 or Superose 6 (Amersham Pharmacia Biotech) with a buffer containing 25 m M Tris-HCl (pH 8.0) and 150 m M NaCl. SIV gp160e (V1V2) and gp160e (V1V2V3) proteins were purified following the same procedure.

SIV gp160e expressed from insect cells was purified by metal chelate affinity chromatography with ProBond resin (Invitrogen). Concentrated insect cell culture supernatants were immediately changed into 1 column buffer (25 m M sodium phosphate (pH 8.0), 250 m M NaCl) in a ProFlux M 12 flow filtration system to remove small molecules in the medium that interfere with the binding of His-tagged SIV gp160e to the nickel column. After centrifugation at 5000 rpm in a JA-14 rotor (Beckman) for 15 min to remove insoluble materials, imidazole was added to the final concentration of 15 m M to reduce nonspecific binding to the resin. Batch binding was then performed at 4 °C for 3 h. After the column was packed (about 5-ml bed volume), the beads were washed by 100 ml of 1 column buffer containing 15 m M imidazole, followed by further washing with 50 ml of 40 m M imidazole in 1 column buffer. The protein was eluted with 300 m M imidazole in 1 column buffer. The fractions were analyzed by SDS-PAGE. The fractions containing SIV gp160e were pooled, concentrated, and further purified by gel filtration chromatography on Superdex 200 or Superose 6 (Amersham Pharmacia Biotech) with a buffer containing 25 m M Tris-HCl and 150 m M NaCl. SIV gp160e (V1V2) and gp160e (V1V2V3) proteins were purified following the same procedure.

N-terminal analyses of purified proteins were carried out by the HHMI biopolymer facility.

Chemical Cross-linking and Analytical Ultracentrifugation- For chemical cross-linking experiments, SIV gp160e protein was dialyzed extensively against PBS. In 20- l reactions, SIV gp160e (1 mg/ml) was incubated with ethylene glycol bis(succinimidylsuccinate) (EGS; Pierce) at concentrations of 0.06, 0.18, 0.55, 1.67, and 5 m M respectively, on ice for 30 min. The reactions were then quenched by adding 5 l of 100 m M Tris-glycine (pH 7.0) and incubated at room temperature for 45 min. The cross-linked products were analyzed by SDS-PAGE. Cross-linked phosphorylase *b* (Sigma) was used as an SDS-PAGE molecular weight standard.

Analytical ultracentrifugation was performed on a Beckman XL-A analytical ultracentrifuge at 4 °C. Experiments were performed at concentrations of 1.6 M , 3.3 M , and 6.5 M protein and centrifuged at a rotor speed of 6000 rpm. Data were fitted to a single-species model. The protein partial specific volume and solvent density were calculated according to Laue *et al.* (49). Briefly, all glycans in gp160e from CHO-Lec3.2.8.1 cells were found to be (GlcNAc) $_{2}$(Man) $_{M}$ by mass spectroscopy using previously described methods (45), and assuming that all 25 potential glycosylation sites are occupied, the partial specific volume of gp160e was calculated to be 0.70 ml/g.

Binding of gp160e to CD4 and Antibody Fab Fragments- Four-domain sCD4 expressed from CHO cells, affinity-purified, and sized by Superdex 75 gel filtration was kindly provided by Dr. Yi Xiong. Mono-

FIG. 1. **Constructs for expression of the ectodomain, gp160e, of the SIV envelope glycoprotein in mammalian and insect cells.** Schematic representations for the expression constructs, pSIV-M (44), pNES1 V1V2V3, pFBSIV-His1, and pFBSIV V1V2V3-His1, are shown. In all cases, the posttranslational cleavage sites (residues 512 and 523) between gp120 and gp41 have been mutated as shown to prevent cleavage and dissociation of gp120. pNES1 V1V2V3 has a shorter tissue plasminogen activator leader sequence than pSIV-M, while both pFBSIV-His1, and pFBSIV V1V2V3-His1 use the honeybee melittin (*HM*) secretion signal. The cleavage sites after the leader sequences were confirmed by N-terminal sequencing of the expressed products and are indicated by *arrows* beneath the sequences. The sequences where the V1, V2, and V3 segments were deleted and replaced with a GAG linker (in *italics*) are shown for pNES1 V1V2V3 and pFBSIV V1V2V3-His1. The sequences where the transmembrane segment of gp41 was truncated are also shown for all constructs. Leader sequences are shown in normal type, and SIV gp160 sequences included in the expression constructs are in *boldface type* and *underlined*; sequences not included are printed with a *strike-through*.

clonal antibodies were purified from cell supernatants of hybridomas growing in roller bottles using a 5-ml GammaBind Plus Sepharose (Amersham Pharmacia Biotech) affinity column. Fab fragments were produced as described (45), from the neutralizing monoclonal antibodies KK9 (46), 17A11 (a kind gift of J. Hoxie), and 9G3. [3] Purified SIV gp160e was incubated at room temperature for 1 h with CD4 or Fab fragments. The complexes were separated from excess unbound CD4 or Fabs by a gel filtration chromatography on Superose 6 (Amersham Pharmacia Biotech). Molecular weights were calculated based on a standard curve plotted from the elution volumes of known proteins. Peak fractions were verified to contain both gp160e and CD4 or Fabs by SDS-PAGE.

Circular Dichroism Spectroscopy- CD spectra were recorded at 25 °C using SIV gp160e at a concentration of 0.56 mg/ml in PBS with a circular dichroism spectrometer model 62DS (Aviv). The molar ellipticity [ ] was monitored as the average of 12 scans with 0.5-nm bandwidth and 1.0-nm wavelength increments from 195 to 260 nm. The spectra were corrected with a base line obtained using buffer alone under the same conditions.

Enzymatic Deglycosylation of SIV gp140- Endoglycosidase H (Endo H) digestion was carried out using 20 g of the SIV gp160e expressed in CHO-Lec3.2.8.1 cells and 500 units of Endo H (New England Biolabs) for each reaction at room temperature under native and denaturing conditions with buffers supplied by the manufacturer. Digestion was then further optimized at various pH values, since low pH may interfere with complex formation of SIV gp160e with the Fabs that were used to prevent aggregation after deglycosylation. Digestion was found to be complete in 30 min at 25 °C under pH 6.5. To deglycosylate the complexes of SIV gp160e and Fab fragments, the purified SIV gp160e was incubated with 17A11, 9G3 Fabs in PBS for at least 2 h at room temperature, followed by adjusting the pH to 6.5 with 100 m M PIPES (pH 6.5). The complexes were then treated with Endo H for 4 h and purified by a gel filtration chromatography on Superose 6. The peak fractions were pooled, concentrated, and analyzed by SDS-PAGE in an 8% gel.

[4] M. Kim and E. L. Reinherz, unpublished results.

RESULTS

Expression of SIV gp160e in Insect and CHO Lec3.2.8.1 Cells- The ectodomain of the SIV envelope glycoprotein, gp160e, was expressed as a secreted protein in both insect and CHO Lec3.2.8.1 cells, by adding a secretion signal (leader)

FIG. 2. **Gel filtration chromatography of purified SIV gp160e expressed from CHO-Lec3.2.8.1 and insect cells.** The SIV gp160e proteins were purified from supernatant of a CHO cell line that expresses the gp160e by mAb 17A11 affinity chromatography (*A*) or from supernatant of insect cells infected with recombinant baculoviruses expressing gp160e by metal chelate affinity chromatography (*B*). The purified proteins were then resolved by gel filtration chromatography using a Superose 6 column. Molecular masses were calculated based on a standard curve plotted using known proteins, which include thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), and bovine serum albumin (67 kDa). Peak fractions were pooled and analyzed by SDS-PAGE (*inset*). Similar experiments using an analytical Superdex 200 column gave the same results (not shown).

sequence 5 to the coding region and terminating the gene just 5 to the transmembrane anchor sequence in gp41 (Fig. 1). Two basic residues at the primary (Lys-523) cleavage site and a secondary (Arg-512) cleavage site between gp120 and gp41 were replaced by glutamic acids, as described previously (44), to prevent cleavage of the gp160e precursor and to avoid shedding of gp120 that had been observed in native constructs. Variants were also expressed with only the V1 and V2 segments deleted (not shown) or with the V1, V2, and V3 segments deleted (Fig. 1). Such deletion variants of HIV-1 gp120 have been shown to retain CD4 binding activity (1, 38-40).

SIV gp160e molecules were secreted into the culture media at levels of 0.5-1 mg/liter from Hi-5 insect cells and 2-4 mg/liter from CHO Lec3.2.8.1 cells.

Purification and Size Determination by Gel Filtration Chromatography- SIV gp160e secreted from CHO Lec3.2.8.1 cells was purified from culture fluid by immunoaffinity chromatography using the monoclonal antibody 17A11 (a gift from J. Hoxie). The protein was eluted from the affinity column by low pH (3.0) and immediately neutralized. Peak fractions were concentrated and further purified by gel filtration chromatography. The purified gp160e eluted from the sizing column (Superose 6) with an apparent molecular weight of approximately 440,000, when compared with standard proteins (Fig. 2 *A*). The relatively sharp gel filtration profile suggests that the molecule with standard proteins (Fig. 2 *B*). The relatively sharp gel filtration profile again suggests that the molecule is reasonably monodisperse. Polydispersity of this sample was also approximately 20%, as measured by dynamic light scattering. Analysis by SDS-PAGE showing a single prominent protein band at an apparent molecular weight of approximately 120,000 indicates a high level of purity (Fig. 2 *B*). Again, the ratio of the monomer molecular weight to the apparent molecular weight of the secreted molecular species indicates that the molecule is an oligomer.

CD spectra of SIV gp160e recorded at 25 °C indicate that the protein is folded and contains a mixture of secondary structures (Fig. 3).

Evidence from Chemical Cross-linking and Analytical Ultracentrifugation That gp160e Is Trimeric- The oligomeric structure of the soluble ectodomain of SIV gp160e was examined by both chemical cross-linking and sedimentation equilibrium by analytical ultracentrifugation. Purified SIV gp160e (1 mg/ml) was incubated for 30 min with the cross-linking reagent EGS at a series of EGS concentrations from 0.06 to 5 m M. The reaction products were analyzed by SDS-PAGE (Fig. 4 *A*). At the lowest concentration of cross-linker, three protein bands are evident, migrating at apparent molecular weights corresponding to one, two, and three gp160e polypeptide chains (see legend to Fig. 4 *A*). As the concentration of cross-linking reagent is increased,

is reasonably monodisperse. Polydispersity is approximately 20% measured by dynamic light scattering. Analysis by SDS-PAGE showing a single protein band at an apparent molecular weight of approximately 120,000 indicates a high level of purity (Fig. 2 *A*). The gp160e monomer molecular weight from SDS-PAGE (120,000) is between one-third and one-fourth of the gp160e oligomer apparent molecular weight (440,000) measured by gel filtration chromatography, indicating that the soluble gp160e molecule is oligomeric.

SIV gp160e secreted from Hi-5 insect cells had been expressed with a C-terminal hexahistidine tag. This gp160e was purified from cell culture fluid by metal chelate chromatography on a nickel resin. The protein was eluted with buffer containing 300 m M imidazole, concentrated, and further purified by gel filtration chromatography. The purified gp160e eluted from the sizing column (Superose 6) with an apparent molecular weight of approximately 440,000 when compared

[3] A. Dessen, W Weissenhorn, J. Skehel and D. Wiley, unpublished results.

the apparent trimer band increasingly dominates. Some faint higher molecular weight bands are observed at higher cross-linker concentrations, but their mobility is too low to represent tetramers, and they presumably represent minor intertrimer cross-linking at high cross-linker concentrations.

The molecular weight of the soluble ectodomain of SIV gp160e was measured by sedimentation equilibrium. Measurements were made at three protein concentrations and one rotor speed. The results from one experiment are shown in Fig. 4 *B*. No systematic deviations were found from a single-species model at the concentrations studied. The partial specific volume of gp160e, required for the buoyancy correction, was calculated from the amino acid composition and the carbohydrate content (see "Experimental Procedures") (49). The resulting measured molecular weight of SIV gp160e of 340,000 is almost exactly 3 times the monomer molecular weight calculated (110,000) from the sequence and carbohydrate content or estimated by SDS-PAGE (120,000).

Complexes of gp160e with CD4 and Fab Fragments of Neutralizing Monoclonal Antibodies- The purified, soluble ectodo-

FIG. 3. **CD spectrum of SIV gp160e.** CD spectra were recorded at 25 °C with a protein concentration of 0.56 mg/ml in PBS. The spectra were corrected with base-line spectra recorded from buffer alone under the same conditions.

main of the HIV-1 receptor human CD4 (4-domain CD4), expressed as a secreted protein in CHO cells, was incubated with purified SIV gp160e to determine its receptor binding activity. Excess CD4 was removed from the gp160e-CD4 complex by gel filtration chromatography (Fig. 5). Analysis of the peak fractions from the gel filtration column by SDS-PAGE indicates that the pure gp160e binds CD4 (Fig. 5).

SIV gp160e binds 13 mAbs raised against SIV gp160 or against SIV-infected cells (mAb 17A11); the mAbs have been mapped to various epitopes: V1, V2, V3, and V4 on gp120 and others on gp41. [2] Complexes of SIV gp160e with Fab fragments from either of the neutralizing mAbs KK9 (which recognizes the V3 and V4 segments of gp120) or 9G3 (which recognizes gp41) were prepared by incubating gp160e with a molar excess of the Fab and removing the excess Fab by gel filtration chromatography. Analysis of the peak fractions from the gel filtration column indicated that both Fabs bind the gp160e molecule. The change in apparent molecular weight estimated from the elution volume of molecular weight standards is approximately 150,000, suggesting that more than one and probably three Fab fragments bind per gp160e oligomer (Fig. 6).

Complexes of gp160e (V1V2V3) with Fab Fragments of Neutralizing Monoclonal Antibodies- A SIV gp160e variant,

FIG. 4. **Chemical cross-linking and analytical ultracentrifugation indicate that SIV gp160e is trimeric.** *A*, SIV gp160e protein was cross-linked at various concentrations of EGS (ethylene glycol bis(succinimidylsuccinate)) as indicated by numbers about the lanes. The cross-linked products were analyzed by SDS-PAGE in a 3.5% gel. The molecular weight standard for SDS-PAGE was cross-linked phosphorylase *b* (Sigma), which includes monomer (97 kDa), dimer (194 kDa), trimer (292 kDa), tetramer (389 kDa), pentamer (487 kDa), and hexamer (584 kDa) species. The molecular masses of monomer, dimer, and trimer bands (Fig. 4 *A*) of SIV gp160e were determined from a plot of the logarithm of molecular mass of the standards against relative mobility (not shown). The bands marked *monomer*, *dimer*, and *trimer* in the *0.06 m M EGS* lane are apparently 123 kDa, 234 kDa (246 calculat-

gp160e (V1V2V3), which has the V1, V2, and V3 segments deleted, also formed complexes with Fab fragments from neutralizing mAbs. The Fab complexes with gp160e (V1V2V3) were purified from the excess Fabs by gel filtration chromatography (Fig. 7). Similar complexes of gp160e (V1V2) with Fabs have been purified (data not shown).

Deglycosylation of SIV gp160e Expressed in CHO-Lec3.2.8.1 Cells- Endo H digestion at room temperature under native and denaturing conditions was used to remove sugar residues from SIV gp160e. Digestion was complete in 30 min at 25 °C, pH 5.5, as judged by the similar increase in mobility on SDS-PAGE of gp160e samples deglycosylated under native and denaturing conditions (Fig. 8 *A*). Deglycosylated SIV gp160e was insoluble.

To prepare soluble, deglycosylated SIV gp160e required that complexes of SIV gp160e and Fab fragments (17A11 or 9G3) be incubated with endoglycosidase H for 4 h. This treatment yielded the same level of deglyscolation as digestion of free gp160e or denatured gp160e, as judged by mobility of the digested gp160e on SDS-PAGE (data not shown). The resulting deglycosylated gp160e-Fab complexes were soluble and monodisperse. These complexes were purified from the excess, unbound Fab by gel filtration chromatography (Fig. 8 *B*).

ed), and 346 kDa (369 calculated), respectively. *B*, analytical ultracentrifugation was performed on a Beckman XL-A analytical ultracentrifuge at 4 °C. The data shown were collected with the protein at concentration of 1.6 M and rotor speed of 6000 rpm. Data sets were fitted to a single species model, and protein partial specific volume and solvent density were calculated according to Laue *et al.* (49). The molecular mass determined is 340 kDa.

DISCUSSION

We have prepared a soluble oligomer of the envelope glycoprotein of SIV in the single chain, precursor state before cleavage into gp120/gp41, in an attempt to capture the native conformation of the envelope glycoprotein before CD4 receptor binding. The expression systems and purification developed provide biochemical quantities of pure protein for use in the study of the functions and structure of the glycoprotein. Following Rhodes and colleagues (44), we have used the tissue plasminogen activator leader sequence to induce secretion of the glycoprotein oligomer from mammalian cells, but we have used a variant cell line, CHO-Lec3.2.8.1 (50), which *N*-glycosylates with only a single, simple oligosaccharide, to reduce heterogeneity in the product and to permit full enzymatic deglycosylation by Endo H. This strategy was previously shown to

FIG. 5. **Purification of a CD4 and SIV gp160e complex by a gel filtration chromatography.** Purified SIV gp160e was incubated with four-domain soluble CD4 from CHO cells (kindly provided by Dr. Yi Xiong). The complexes were separated from excess unbound CD4 by a gel filtration chromatography on a Superdex 200 column. The peak fractions were pooled, concentrated, and analyzed by SDS-PAGE in an 8% gel. *Lane 1*, peak eluted at 10 ml; *lane 2*, peak eluted at 14.5 ml.

FIG. 7. **Purification of complex of an SIV gp160e deletion variants with V1, V2, and V3 segments deleted, gp160e (V1V2V3), and Fab fragments.** Purified SIV gp160e (V1V2V3) expressed from pFBSIV V1V2V3-His1 was incubated with Fab fragment generated by papain digestion of the neutralizing monoclonal antibody 17A11 (a gift from J. Hoxie), which recognizes an epitope on gp120. The complex was separated from excess unbound Fabs by a gel filtration chromatography on a Superose 6 column. The peak fractions were pooled, concentrated, and analyzed by SDS-PAGE in an 8% gel. *Lane 1*, peak eluted at 14 ml; *lane 2*, peak eluted at 18 ml.

forming complexes with Fab fragments from neutralizing mAbs (Fig. 8 *B*).

We have also expressed the molecule in insect cells, using the honeybee melittin leader sequence to induce secretion. Both molecules purified from the culture fluids of mammalian and insect cells appear to be in a native conformation judging from their abilities to bind the soluble ectodomain of the viral receptor CD4 (Fig. 5; not shown for insect cells) and to be recognized by a number of conformationally sensitive neutralizing monoclonal antisera [2] (Fig. 6). The gp160e from mammalian cells was purified by immunoaffinity chromatography requiring a low pH elution step, while a hexahistidine tag on the gp160e from insect cells permitted purification by metal chelation chromatography. Both methods produced proteins with the same properties, suggesting that the purified protein has a single conformation that is robust to such treatments.

Following Rhodes and colleagues (44), we eliminated the primary and secondary cleavage sites between the gp120 and gp41 segments by replacing two basic residues with glutamic acids. In experiments with SIV gp160e molecules produced in

FIG. 6. **Purification of complexes of SIV gp160e with Fab fragments derived from neutralizing monoclonal antibodies by gel filtration chromatography.** Purified SIV gp160e was incubated with Fab fragments (as shown 9G3 in *A*, KK9 in *B*) generated by papain digestion from either the KK9- (46) or 9G3 [2]-neutralizing mAb, which recognize epitopes on gp120 and gp41, respectively. The complexes were separated from excess unbound Fabs by gel filtration chromatography on a Superose 6 column. The apparent molecular mass was calculated based on a standard curve plotted using the elution times of known proteins. The peak fractions were pooled, concentrated, and analyzed by SDS-PAGE in an 8% gel. *Lane 1*, peak eluted at 13 ml; *lane 2*, peak eluted at 18 ml.

work well for other heavily glycosylated surface protein (45). We have confirmed that the protein purified from the culture fluid of the CHO cells can be completely deglycosylated by enzyme treatment (Fig. 8 *A*), and we have shown that deglycosylated oligomers can be stabilized against aggregation by CHO cells without these mutations, we had observed spontaneous cleavage between gp120 and gp41 and some shedding of gp120 during purification. [4] With the two mutations present, no cleavage between gp120 and gp41 has been observed (Fig. 2, *inset*), permitting the production of quantities of a stable oligomeric ectodomain.

The finding that the transmembrane anchor-containing subunits of the lenti- and retrovirus, HIV-1, SIV, MoMuLV, and HTLV-1 are all trimeric (21, 51-53) and some, but not all, of the earlier attempts to define the oligomeric state of HIV gp160 (27, 28) suggested that SIV gp160e was trimeric. We add evidence for a trimeric state from chemical cross-linking and from a direct molecular weight determination on the gp160e. Chemical cross-linking generates products that form three bands on SDS-PAGE, which migrate at apparent molecular weights that are 1, 2, and 3 times that of the gp160e monomer as determined by SDS-PAGE (120,000) (Fig. 4 *A*). This "ladder" is a direct indication of the presence of a trimer of gp160e. Earlier chemical cross-linking experiments on full-length HIV gp160 have been interpreted to favor trimeric or tetrameric states. The

[4] A. Dessen, W. Weissenhorn, J. Skehel and D. Wiley, unpublished results.

FIG. 8. **Enzymatic deglycosylation of SIV gp160e and purification of a complex of deglycosylated SIV gp160e and Fab fragments by gel filtration chromatography.** *A*, Endo H digestion was carried using the SIV gp160e expressed in CHO-Lec3.2.8.1 cells. *Lane 1*, untreated SIV gp160e; *lane 2*, the Endo H used in this assay; *lane 3*, SIV gp160e treated with Endo H under native condition; *lane 4*, SIV gp160e treated with Endo H under denaturing condition. *B*, purified SIV gp160e was incubated with 17A11 and 9G3 Fabs generated by papain-digestion of 17A11 and 9G3 monoclonal antibodies, respectively. The complexes were treated with Endo H under native conditions and purified from the excess Fabs by a gel filtration chromatography on a Superose 6 column. The peak fractions were pooled, concentrated, and analyzed by SDS-PAGE in an 8% gel. *Lane 1*, untreated gp160e control; *lane 2*, peak eluted at 12.5 ml; *lane 3*, peak eluted at 18 ml.

experiments on the pure ectodomain (Fig. 4 *A*) clearly indicate a trimer.

An independent method of establishing the oligomeric state is to measure the molecular weight of the monomer and the oligomer. The oligomer molecular weight was measured as 340,000 by equilibrium sedimentation, a method that yields a value independent of molecular shape or hydrodynamic radius. The monomer molecular weight measured by mass spectrometry (100,000) or calculated from the amino acid sequence and carbohydrate content (110,000) is almost exactly one-third of the oligomer molecular weight, again indicating a trimeric structure. The centrifugation data show no indication of a monomer-oligomer equilibrium, demonstrating that the oligomeric species is stable under the conditions studied (Fig. 4 *B*).

The similarity of the sequences and functions of the SIV and HIV envelope glycoproteins suggest that they will have very similar structures. Both bind the same receptor molecules and undergo conformational alterations leading to membrane fu-

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sion/viral entry activities. The conformations of the SIV and HIV-1 gp41 ectodomains, determined from molecules expressed without the gp120 subunit are essentially identical (22, 23, 51, 54-56). The sequence of SIV gp160 is over 50% identical to that of HIV-2, and that of HIV-2 to HIV-1 is about 30%. Molecules related at this level have very similar structures, which normally allow structure/function insights to be drawn from one to the next.

The availability of methods to produce and purify mg quantities of SIV gp160e should improve the possibilities for determining the structure of the precursor state of a lentivirus envelope glycoprotein closely related to HIV. The only structure of a precursor viral glycoprotein currently known is that of HA0 of influenza virus (57). In that case, only 19 residues in the vicinity of the cleavage site differ in structure between the precursor and the cleaved (HA1/HA2) structure. It remains to be seen whether the structure of gp160e will resemble as closely that of proteolytically processed gp120-gp41 complex.

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Dr Rod Daniels team:

- Variation in HIV *env*-genes and their encoded proteins: studies on the structural, functional, biochemical and immunologic properties of the glycoproteins
- Influenza studies

Variation in HIV *env*-genes and their encoded proteins: studies on the structural, functional, biochemical and immunologic properties of the glycoproteins

HIV is the etiological agent of AIDS and in common with other retroviruses a high degree of variation exists between strains. This variation is particularly high in the *env*-gene and its product, a highly glycosylated protein (gp160) which is subsequently processed to gp120 and gp41. These proteins play major roles in the infectious cycle due to receptor binding and membrane fusion activities and are a major target for all arms of the host immune system. Our work is concentrated on *env*-gene products with the aim of understanding the mechanisms of the above functions. To achieve these objectives our work is focused in three main areas:

1. Reference and surveillance studies

By working with well documented cases of HIV infection we are attempting to relate *env*-gene variation to the biology of the

development of AIDS. To assist in this numerous collaborations have been established to supply the required clinical samples in terms of, e.g. vertical transmission, rate of disease progression, tropism for particular cell types. An efficient system for the PCR rescue of *env*-genes and assessment of their expression competence, prior to sequencing, has been established. The glycoprotein translation products are analysed using the Programme "Variate", written by the NIMR HIV group, together with commercial packages to identify amino acid substitutions which may be relevant to the biology of the disease. Such analyses have enabled us to identify signature patterns in the glycoprotein of HIV-1 subtype B strains spreading in the population of the Republic of Korea (ROK). To assess the functionality of such glycoproteins, receptor-binding and fusion assays have been developed and an infectious molecular clone *env*-gene cassetting system developed to allow study of glycoprotein function within a constant HIV-1 genetic background.

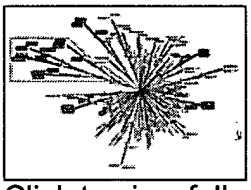

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Figure 1: *Env*-gene Phylogeny of HIV-1 subtype B strains circulating in the ROK

80% of HIV infections in the ROK are caused by HIV-1 subtype B and of these 80% fall within a distinct subclade compared to other subtype B viruses circulating in other countries. Sequences recovered from Korean patients are highlighted in yellow and those within the distinct subclade are indicated by the red enclosure.

2. Glycoprotein structural and

functional studies

Our main priority is to produce and characterise glycoprotein suitable for crystallographic analysis. Using the CHO-pEE14tpa expression system with *env*-genes derived from our reference and surveillance studies we have produced gp120s that form small crystals but, to date, these have not diffracted well. The high degree of glycosylation (50% by weight) is considered to be a major factor in the poor diffraction. This is being addressed by partial/complete enzymatic deglycosylation. Recently we have shifted focus to the expression of soluble forms of gp140 which have elements built into them that should enhance stability of trimer formation. These are being expressed in the CHO-Lec cell line which is defective in oligosaccharide maturation thereby making removal of carbohydrate easier. Information and reagents generated are relevant to the design and formulation of vaccines, prophylactic and therapeutic, and anti-HIV drugs directed against glycoprotein functions.

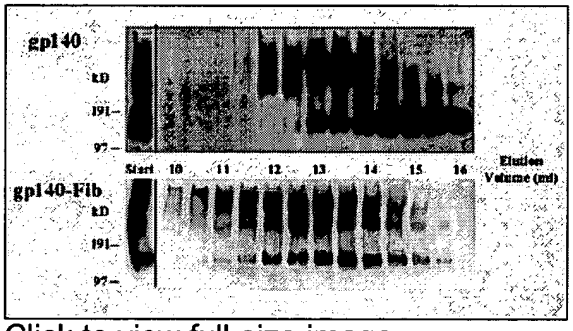

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Figure 2:

Oligomeric state of HIV-1 glycoprotein (gp140) analysed by gel filtration chromatography

Lane 1 shows start material, thereafter fractions from gel filtration are run. Whilst the gp140 sample contains some trimeric material (>440kD: <14ml elution volume) addition of the Fibritin 'foldon' domain results in the majority of the gp140-Fib sample being trimeric.

3. Virus isolation, neutralisation and vaccine studies

Macrophage-tropic HIV strains play a major role in virus transmission but established systems for virus isolation, human cell-lines and cytokine-activated peripheral blood mononuclear cells (PBMC), often do not support replication of such strains or they are produced at very low virus titre. We have established that *Herpesvirus saimiri* transformed human T-cells (HVS T-cells) support high-titred replication of HIV-1 and -2, give higher titres of macrophage-tropic viruses than the other two systems and give efficient rescue of viruses from patients enrolled in a long-term non-progressor cohort. The HVS T-cells are suitable for use in neutralisation assays and their stability compared to PBMC preparations should allow better standardisation of such assays. These will be important in assessing the efficiency of new vaccine constructs based on virus-like particles (VLPs) being developed in collaboration with members of an MRC Co-operative.

Overall, the above approaches integrate to provide a thorough study of HIV glycoproteins.

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Influenza studies

Contributions have been made to the understanding of interactions between

neutralising monoclonal antibodies and influenza haemagglutinin. In addition, sensitive RT-PCR techniques, developed for the study of HIV, are being adapted to allow identification/characterisation of the causative agent of the 1918 influenza pandemic and viruses circulating in the human population pre-1933. Formalin-fixed paraffin-embedded tissue sections and tissues recovered from burials in permafrost provide the sources of influenza RNA for these studies. The same techniques have been used to study the tissue distribution of A/Puerto Rico/1/34 (H1N1) and B/Lee/1/40 viruses in a murine model of Reye's syndrome.

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Back to top ^

Drugs & Medications
News Update
"HIV Vaccines--Where Are We Going?"

"HIV Vaccines--Where Are We Going?"

Nature Medicine--Vaccine Supplement (05/98) Vol. 4, No. 5, P. 532; Heilman, Carole A.; Baltimore, David

In a vaccine supplement to Nature Medicine, Carole A. Heilman-- of the National Institutes of Allergy and Infectious Diseases--and David Baltimore--head of the California Institute of Technology--review current HIV vaccine technology and progress. HIV vaccine research focuses primarily on two systems: the humoral immune system and cellular immune response. The surface protein Env--a processed, mature trimer which is comprised of gp160 co-proteins, which are cleaved into gp120 and gp41 proteins--has become the main target of antibody-based vaccine candidates. Research has centered on eliciting high affinity, broadly reactive antibodies that can contain the virus. Some vaccine studies aim to design candidates which increases cellular immunity through the development of cytotoxic T lymphocytes (CTLs) against HIV encoded proteins. Scientists will soon begin trials in Uganda to determine if individuals from different genetic backgrounds who are exposed to viruses of different clades will recognize CTL epitopes in a vaccine candidate for a given clade. Another vaccine design approach has been the creation of a live, attenuated HIV vaccine. This method has been successful in the development of vaccines against other viruses and in some animal models, but concerns exist about a possible reversion of the virus causing disease. Scientists are also exploring the possibility of creating a vaccine which combines the humoral and cellular responses. This "prime-boost strategy" appears to have an additive effect in the immune response. Finally, new model systems are in development which could help testing for vaccine candidates. A "chimera" virus, called SHIV, has been designed to allow HIV-based envelope vaccines to be evaluated in an SIV model. Scientists have also designed transgenic mice and rabbits which express human CD4 cells, allowing for the replication of low-levels of HIV, which could serve as a new animal model.

Previous Back

|| Back to the Drug/News Archive Page || Back to the Medication Page ||